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Fasudil (a Rho Kinase Inhibitor) Specifically Increases Cerebral Blood Flow in Area of Vasospasm After Subarachnoid Hemorrhage

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1. Introduction

Subarachnoid hemorrhage due to a rupture of cerebral aneurysm is a severe disease with morbidity and mortality. Although, if patients' conditions are fair before surgery, they are rather safely operated by either clipping or coiling, vasospasm remains as a major complication of this disease. There are still many patients who suffer from vasospasm causing neurological deficits. Both strong vasoconstriction and inflammation are involved in the pathphysiological mechanism of vasospasm. In 1992 we had reported specific effects of a vasodilating drug "fasudil" in the treatment of vasospasm, but mechanisms how fasudil ameliorated vasospasm had not been clearly understood as it is today.
RhoA/Rho kinase had been found in 1996 and was revealed to act as molecular on-off switches that control multiple signaling pathways. Upregulated Rho kinase is known to be involved in various diseases from vascular disease to cancer. In cerebral vasospasm, upregulated Rho kinase was found to be involved in many aspects, such as increased calcium sensitivity, reduced production of nitric oxide, migration of inflammatory cells and their production of superoxide anions and increased blood viscosity. Interestingly, fasudil was found to specifically increase cerebral blood flow in the area with vasospasm. In the present paper pathophysiological mechanism of vasospasm and effects of fasudil are reviewed and mechanisms why fasudil increases cerebral blood flow in the area with vasospasm without so much changing that of normal flow area will be discussed.

2. Cerebral vasospasm following subarachnoid hemorrhage

Cerebral infarction due to delayed vasospasm is still the leading cause of a poor postoperative outcome of patients with a ruptured cerebral aneurysm especially if we consider deficits in higher neurological functions such as cognitive functions. Several days after a subarachnoid hemorrhage (SAH), blood vessels begin to be contracted by substances eluted from the blood clot such as oxyhemoglobin, endothelin, amines and many other chemical substances. Most of the patients show contraction of the blood vessels (angiographic spasm). In about one third of the patients, signs of neurological deficits appear (symptomatic spasm) on an average of day 7 after the hemorrhage (Bederson et al., 2009, Shibuya et al., 1992). Patients may even die of severe spasm, especially due to vasospasm of arteries supplying basal part of the brain: hypothalamus and brainstem. A representative case of a patient with severe vasospasm is shown in Fig. 1.

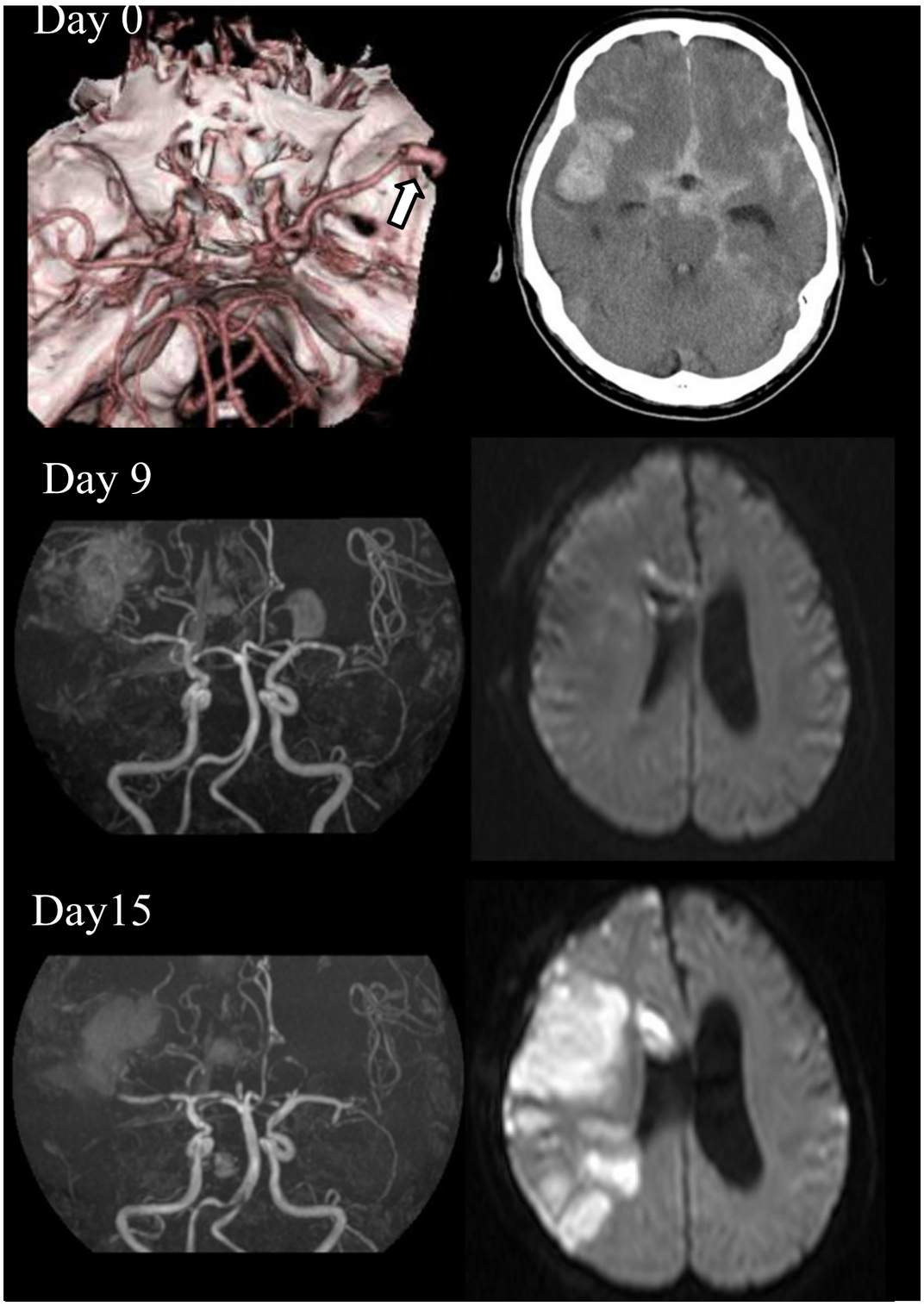

Fig. 1. Representative case of a patient with severe vasospasm.
Patient is a 70y/o male with a past history of prostatic cancer, hypertension and diabetes mellitus. He had a sudden onset of severe headache and lost consciousness two times at

home. He was slightly drowsy and disoriented (Hunt & Hess grade III) with mild weakness in the left arm and leg. A head computed tomography (CT) (upper right) showed a diffuse subarachnoid hemorrhage and a large hematoma in the right Sylvian fissure. A 3-D CT angiogram (upper left) showed a 10mm long aneurysm at the bifurcation of the right middle cerebral artery (MCA) (arrow). The aneurysm was clipped and subarachnoid space was washed with urokinase on the same day. He smoothly recovered from surgery and he was treated routinely postoperatively to prevent vasospasm with careful management of blood pressure, water and electrolytes balance. Fasudil 30mg (i.v./30min, t.i.d.) was started on day 1. His postoperative course was smooth with clear consciousness and a mild left hemiparesis.
A routine checkup, on day 9, by a magnetic resonance angiography (MRA, middle left) showed a moderately severe vasospasm in the right MCA, a segmental vasospasm in the left MCA and proximal portion of the right anterior cerebral artery (ACA). Diffusion weighted magnetic resonance image (DWI) showed no abnormality (middle right). His blood pressure was elevated with dopamine and daily dose of fasudil was increased to 60mg (i.v., t.i.d.) to prevent development of further neurological deficits.
However, the next day (day 10), his left hemiparesis deteriorated and he became drowsy. MRA on day 15 showed that vasospasm in bilateral MCAs progressed. Especially, distal branches of the right MCA were hardly seen. Segmental vasospasm appeared in the proximal portion of the right MCA, left ACA and distal portion of the vertebral arteries (lower left). However, vasospasm in the proximal portion of the right ACA improved. DWI on the same day showed an infarction in the right MCA territory (lower right). In spite of deterioration of vasospasm on MRI and MRA on day 15, he began to recover his consciousness the same day. Although he was communicable and could eat by himself, his left hemiplegia did not improve and he was discharged to a rehabilitation hospital. Now, two years after the onset, he is bed ridden and taken care at his home.

Vasospasm is not a simple contraction of blood vessels but it is complex pathological phenomena consisting of abnormal contraction of blood vessels which is not easily relaxed by usual calcium antagonists and inflammation. Tissue damage is seen in vascular endothelium and smooth muscle cells in the medial wall caused by free radicals released from inflammatory cells. Decreased production of nitric oxide (NO) is also contributing to both contraction and tissue damage. Rho kinase has been found to be deeply implicated in the pathophysiology of vasospasm (Miyagi et al., 2000; Sato et al., 2000) and use of a Rho kinase inhibitor: fasudil dramatically improved patients' outcome (Shibuya et al., 1992)

3. Effects of Fasudil, a Rho kinase inhibitor on cerebral vasospasm

Fasudil HCl: (hexahydro-1-5-isoquinolinesulfonyl)-1H-1.4-diazepine HCl, (also called HA1077, AT877, or Eril@) is originally considered to be an intracellular calcium antagonist. By experimental studies in dogs we had found that fasudil dilated spastic arteries without causing systemic hypotension, which could not been shown by any of the previously presented drugs (Takayasu et al., 1986). The effectiveness was also confirmed in patients by a double blind trial (Shibuya et al., 1992). Fasudil showed stronger brain protection from ischemic damage than dilatation of the spastic artery itself, suggesting its possible effects in patients with cerebral infarction as well. Fasudil is now routinely used in Japan for patients with SAH. Zhao et al. (2007) in China showed by a randomized trial that fasudil was

significantly better for vasospasm than nimodipine which was most commonly used in the western countries.

After Rho kinase was found (Kimura et al., 1996), it became clear that upregulated Rho kinase worked unfavorably to the host in many vascular diseases and effects of fasudil on vasospasm mainly depended on its inhibition of Rho kinase. Fasudil was found to inhibit Rho kinase most strongly than any other protein kinases such as protein kinases C, A, and G (Hidaka et al., 2005). Fasudil is metabolized in human to hydroxyfasudil. Both fasudil and hydroxyfasudil are strong inhibitors of Rho kinase, however biological half-life of fasudil and hydroxyfasudil after an intravenous infusion of fasudil in human are 18 min and 6 hours, respectively. Thus major effect is considered to depend on hydroxyfasudil rather than fasudil itself.

Upregulated Rho kinase inhibits relaxation of the contracted blood vessels by inhibiting dephosphorylation of phosphorylated myosin light chain (MLC) either directly or through inhibition of endothelial NO synthase (eNOS). In an experimental model of vasospasm induced by $PGF_2\alpha$, double phosphorylation of MLC, at Thr18 in addition to Ser19, was found. This is considered to be the underlying mechanism of the strong contraction or increased sensitivity to Ca^{++}. Furthermore, fasudil was found to inhibit the second (pathological) phosphorylation at Thr18 of MLC more strongly (IC50: 0.3uM) than the first phosphorylation at Ser19 (IC50: 3uM) (Seto et al., 1991).

4. Fasudil specifically increases rCBF in area with vasospasm

Specific effect of fasudil on cerebral vasospasm has been suggested to depend on its inhibition of the abnormal phosphorylation of MLC. On the other hand, under normal situation, increased intracellular calcium phosphorylates MLC by activating calmodulin and myosin light chain kinase (MLCK) which is relaxed by dephosphorylation of MLC by phosphatase.

In a two hemorrhage canine model of SAH, basilar artery diameter is decreased to about 60% on day 7. Intravenous administration of a calcium antagonist nicardipine (0.1mg/kg, i.v./30min) did not dilate the spastic basilar artery but caused systemic hypotension. While fasudil (HA1077) (0.5~3mg/kg, i.v. /30min) significantly dilated the spastic artery without causing hypotension (Takayasu et al., 1986). It can be explained by specific inhibition of Rho kinase by fasudil. In other words, fasudil dilated spastic artery more specifically than normal or non-spastic arteries.

Specific vasodilating effect of fasudil has been shown by measuring regional cerebral blood flow (rCBF). In patients who had been operated on their ruptured aneurysms, Ueda (2000) compared the effects of fasudil on rCBF using 99mTc-HMPAO with that of nicardipine. Nicardipine (2mg, i.v.) decreased BP and increased pulse rate. It decreased rCBF in the low flow (spastic) area (to -10%, $P<0.05$) without changing rCBF of the normal flow area, suggesting a loss of autoregulation in the spastic area. On the other hand, fasudil (15 mg, i.v.) increased rCBF in the low flow area by 16% ($P<0.05$) without changing that of normal flow area.

Using CT perfusion method in patients with SAH, Ono et al. (2005) have examined changes in the cerebral blood perfusion (CBP) by fasudil (30mg, i.v./30min) in both normal (>40ml/100g/min) and low flow (<40ml) regions due to vasospasm. The mean CBP in the low flow area (34.4±4.7ml) was significantly increased (to 41.0±8.2 ml, $P<0.05$, n=43), whereas the mean CBP of the normal flow region (51.8±7.6ml) did not change after fasudil

(50.4±8.4ml, n=125). We also have shown by using 99mTc-HMPAO that fasudil (30-60mg/i.v./30 min) significantly increased rCBF in the operated side of the brain in patients showing ischemic signs of vasospasm. Such difference was not found in patients without vasospasm (Shibuya et al., 2008).

These data suggest that upregulated Rho kinase is involved in the decrease of rCBF in patients with vasospasm which was specifically improved by a Rho kinase inhibitor fasudil. On the other hand calcium antagonist dilated normal arteries more than spastic arteries leading to a systemic hypotension and a steal phenomenon, a steal of blood from a spastic region to a normal region.

5. Effects of fasudil on cerebral infarction

Rho kinase is also up-regulated in patients with cerebral infarction, both in ischemic brain and in migrated WBCs. It is involved in many aspects of ischemic brain damage caused by migration of inflammatory cells to the ischemic site and their production of free radicals by activated NADPH oxidase. Rho kinase elevates blood viscosity by producing the tissue factor (also called factor III, thrombokinase, or CD142) which triggers the coagulation cascade. Blood viscosity is also elevated by reduced plasticity of RBCs due to polymerization of actin fibers which is induced by activated Rho kinase and protein kinase C (Arai et al., 1993; Brabeck et al., 2003; Feske et al., 2009; Satoh et al., 2010). Effectiveness of fasudil on cerebral infarction has been shown both by experimental (Tsuchiya et al., 1993) and clinical studies (Shibuya et al., 2005). After specific effects of fasudil on cerebral vasospasm and infarction had been shown, it has been tried and showed effectiveness in various kinds of vascular diseases such as coronary ischemia, glaucoma, pulmonary hypertension, chronic kidney disease and so on (Dong et al., 2010, Schmandke et al., 2007).

6. Discussion

6.1 Rho kinase

Rho kinase is the immediate downstream target of RhoA, a small GTP binding protein belonging to Ras, Rho, Rab and Ran subfamilies and acts as molecular on-off switches that control multiple signaling pathways. Inactive form of Rho-GDP is activated by guanine nucleotide exchange factors (GEFs) and Guanine dissociation inhibitors (GDIs) through stimulation by lysophosphatidic acid (LPA) and sphyngosine-1-phosphate (S1P). Active form (GTP and membrane-bound) RhoA is inactivated by GTPase activating proteins (GAPs) to GDP bound form in cytosole. Rho kinase is a serine-threonine protein kinase that are involved in diverse cellular functions including vascular smooth muscle cell (SMC) contraction such as cerebral and coronary vasospasm, atherosclerosis, actin cytoskelton arrangement, cell adhesion, motility and gene expression (Noma et al., 2006).

6.2 Upregulated Rho kinase and increased sensitivity to calcium in vasospasm

Miyagi et al. (2000) showed that RhoA and mRNA of Rho kinase was increased in the basilar artery of SAH rats. Sato et al. (2000) clearly showed, in a two hemorrhage dog model, that Rho kinase was up-regulated with the decrease in basilar artery diameter and with the increase of phosphorylation of myosin binding subunit (MBS) of myosin phosphatase of the basilar artery, all of which were inhibited by a Rho kinase inhibitor Y27632. Activated Rho kinase inhibits MLC phosphatase by phosphorylating its component MBS at Thr697 (Feng et

al., 1999) either directly or through activation of protein kinase C (PKC). PKC activated protein kinase C-potentiated inhibitory protein-17 (CPI-17) by phosphorylating at Thr38 (Koyama et al., 2000). In vasospastic condition, contraction force is increased without changes in intracellular concentration of Ca^{++}. Thus double (sometimes triple) phosphorylation of MLC by upregulated Rho kinase is considered to be the mechanism of so called increased sensitivity to Ca^{++}.

6.3 Involvement of inflammation in vasospasm

Inflammatory cells migrate to vasospasm or infarction sites and cause tissue injury by producing free radicals. When human WBCs were incubated in a Boyden chamber, WBCs migrated through a millipore filter by adding a chemoattractant such as formyl-methionyl-leucyl-phenylalanine (fMLP) to one side of the chamber. This migration was dose dependently inhibited by fasudil (Satoh et al., 1999). When WBCs were incubated with phorbol myristate acetate (PMA), a protein kinase C activator, they produced superoxide anion ($O_2^{-)}$ by NADPH oxidase, which also was dose dependently inhibited by fasudil (Arai et al., 1993). Free radicals such as O_2^- are known to cause structural damage in endothelial cells and SMCs, leading to a decreased production of nitric oxide (NO) by endothelial NO synthase (eNOS).

6.4 Inhibition of NO synthase (eNOS) by Rho kinase

Nitric oxide (NO) plays an important role in the regulation of vascular tone, inhibition of platelet aggregation, suppression of SMC proliferation and prevention of leukocyte recruitment to the vessel wall. Activity of eNOS is controlled by a variety of signals surrounding blood vessels. Laminar shear stress, O_2 tension and transforming growth factor (TGF)β1 can regulate eNOS expression at the transcriptional level. Chronic hypoxia, tissue necrosis factor (TNF)α, thrombin, oxidized low density lipoprotein (LDL) and cellular proliferation are known to regulate eNOS expression at postscriptional level. Shear stress and vascular endothelial growth factor (VEGF) rapidly activated eNOS by phosphorylating at Ser1177. Hypoxia is known to upregulate Rho kinase which inhibits eNOS by phosphorylating at Thr495 (Flemming et al., 2001; Noma et al., 2006; Sugimoto et al., 2007). On the other hand, inhibition of Rho kinase by hydroxyfasudil increased phosphorylation of protein kinase Akt Ser473 and production of NO (Wolfrum et al., 2004). NO relaxes blood vessels by activating guanylate cyclase (which produced cyclic GMP) and protein kinase G, which activated MLC phosphatase by phosphorylating its component MBS at Ser695 (Nakamura & Ikebe, 2007, see also Fig. 2).

Pulmonary hypertension is a fatal disease in which eNOS activity is decreased. When human vascular endothelium was incuvated under hypoxic state of 3% O_2, both expression of mRNA of eNOS and eNOS activity were suppressed. The suppression was ameliorated by Rho kinase inhibitors, botulinus C3 transferase and fasudil (Takemoto et al., 2002). Actually, fasudil showed good results in patients with pulmonary hypertension (Fukumoto et al., 2005).

6.5 Increased blood viscosity in cerebral vasospasm and infarction

Blood viscosity is elevated in patients with acute cerebral infarction (Coull et al., 1991). However, it is not clear if this reflects a pre-existing risk factor or an acute phase response to the stroke itself or both. In rats model of temporary ischemia, by passing a nylon thread

through the carotid artery for one hour and then removed, blood viscosity measured 24 hours later by a cone-plated discometer (at 37.5 rpm), was elevated from a control of 5.31 centipoise (cP) to 6.05 cP. Fasudil (1~10mg/kg) dose dependently inhibited the elevation of blood viscosity (Hitomi et al., 2000). Both production of the tissue factor (Zhang et al., 2007) and Rho kinase-activated polymerization of f-actin are considered to be involved in the increase of blood viscosity which were ameliorated by fasudil（Feske et al., 2009, Nagata et al., 20022Satoh et al., 2010).

RhoA/Rho kinase pathway has been shown to be involved in many other vascular diseases such as angiogenesis, atherosclerosis, cerebral and coronary spasm and infarction, glomerulosclerosis, hypertension, ischemia-reperfusion injury, neointimal proliferation, bronchial asthma, glaucoma and so on. Our current concepts about the Rho-kinase related mechanisms and effects of a Rho kinase inhibitor fasudil in cerebral vasospasm and infarction are shown in Fig. 2.

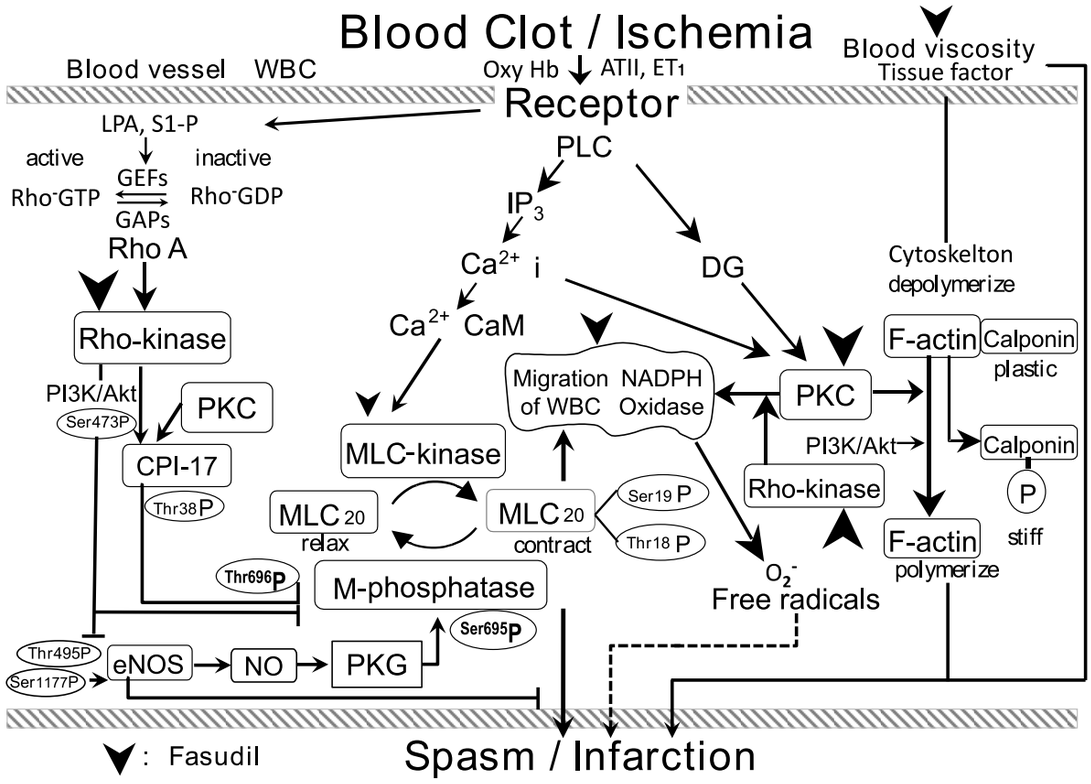

Fig. 2. Rho kinase related mechanisms and effects of fasudil in cerebral vasospasm and infarction

Chemical ligands eluted from subarachnoid blood clot or from ischemic brain such as oxyhemoglobin, angiotensin II and endothelin increase intracellular lysophosphatidic acid (LPA) and sphyngosine-1-phosphate (S1-P) which activate RhoA through activation of guanine nucleotide exchange factors (GEFs) from an inactive GDP-Rho in the cytosole to an active and membrane bound GTP-Rho. Activated Rho kinase contracts blood vessels by inhibiting myosin light chain (MLC) phosphatase by phosphorylating its component myosin binding subunit (MBS) at Thr696 through activation of protein kinase C-potentiated

inhibitory protein-17 (CPI-17). Rho kinase also inhibits relaxation of contracted blood vessels by inhibiting endothelial nitric oxide synthase (eNOS) through inhibition of phosphatidylinositol-3kinase (PI3K)/protein kinase Akt. On the other hand, eNOS is activated by dephosphorylation at Thr495 or phosphorylation at Ser1177 when Rho kinase is inhibited. NO relaxes blood vessels by activating guanylate cyclase and protein kinase G (PKG). PKG activates MLC phosphatase by phosphorylating its component myosin binding subunit (MBS) at Ser695.

On the other hand, migration of inflammatory cells like WBCs and their production of free radicals by NADPH oxidase are stimulated by upregulated Rho kinase and protein kinase C. Rho kinase also increases blood viscosity by producing the tissue factor which triggers the coagulation cascade and also by decreasing plasticity of RBCs. Plasticity of RBCs is decreased when f-actin, consisting cytoskeleton, is polymerized by Rho kinase and protein kinase C.

These adverse phenomena: abnormal contraction of blood vessels, migration of inflammatory cells and their production of free radicals, increase of blood viscosity had all been ameliorated by a Rho kinase inhibitor fasudil which showed in turn that upregulated Rho kinase is involved in each of these sites (see text for references). Arrow head indicates acting points of fasudil.

7. Conclusion

Upregulated Rho kinase is deeply implicated in the complex mechanisms of delayed cerebral vasospasm after a subarachnoid hemorrhage, in both vasoconstriction and inflammation. Double phosphorylation of myosin light chain leading to pathological contraction, suppression of eNOS, production of free radicals are all induced by upregulated Rho kinase. Fasudil improved these situations by mainly inhibiting upregulated Rho kinase, which can explain why fasudil specifically increased cerebral blood flow in the area with vasospasm.

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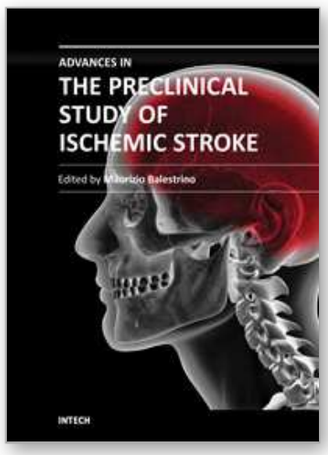

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This book reports innovations in the preclinical study of stroke, including - novel tools and findings in animal models of stroke, - novel biochemical mechanisms through which ischemic damage may be both generated and limited, - novel pathways to neuroprotection. Although hypothermia has been so far the sole "neuroprotection" treatment that has survived the translation from preclinical to clinical studies, progress in both preclinical studies and in the design of clinical trials will hopefully provide more and better treatments for ischemic stroke. This book aims at providing the preclinical scientist with innovative knowledge and tools to investigate novel mechanisms of, and treatments for, ischemic brain damage.

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